JOURNAL OF VIROLOGY, Jan. 1983, p. 215–225
0022-538X/83/010215-11$02.00/0

Vol. 45, No. 1

Physical Analysis of *Autographa californica* Nuclear Polyhedrosis Virus Transcripts for Polyhedrin and 10,000-Molecular-Weight Protein

GALE E. SMITH,[1] JUST M. VLAK,[2] AND MAX D. SUMMERS[1]*

Department of Entomology, Texas A&M University and The Texas Agricultural Experiment Station,[1] College Station, Texas 77843, and Department of Virology, Agricultural University, 6709 PD, Wageningen, The Netherlands[2]

Received 30 August 1982/Accepted 4 October 1982

In *Autographa californica* nuclear polyhedrosis virus-infected cells, polyhedrin, the major structural polypeptide of the viral occlusions, and a low-molecular-weight viral protein with a molecular weight of approximately 10,000 (10K) accumulated to high levels late in infection. Two polyadenylated RNAs 1,200 and 630 bases in size were the most abundant viral transcripts present in the cytoplasm at 48 h postinfection. Evidence is presented that these RNAs were the transcripts for polyhedrin and 10K proteins. The 630-base RNA and five other major polyadenylated RNAs present at 48 h postinfection mapped within or near *A. californica* nuclear polyhedrosis virus *Eco*RI-P. The DNA sequences that code for polyhedrin mRNA were examined by S1 nuclease analysis. The polyhedrin gene contained no detectable introns and mapped at 3,990 to 5,200 base pairs to the right of the origin of the circular *A. californica* nuclear polyhedrosis virus physical map. Heterogeneity at the 5′ end of polyhedrin mRNA was observed by using S1 nuclease analysis with 5′ end-labeled DNA probes.

Autographa californica nuclear polyhedrosis virus (AcMNPV) is the prototype virus of the animal virus family Baculoviridae. Many studies on the molecular biology of baculoviruses are conducted with AcMNPV primarily because it is infectious in many species of cultured insect cells and has a corresponding broad host range in vivo. The latter property also makes AcMNPV potentially useful as a biological pesticide for the control of agriculturally important pests. AcMNPV has a circular duplex DNA genome of about 128 kilobase pairs and about 40 infected-cell-specific proteins have been detected in infected cells (3, 5, 9, 12, 23, 27, 29). Of these, we have mapped the approximate location of 19 AcMNPV genes by hybridization selection and in vitro translation of restriction fragment-specific mRNA (23, 27).

Central to our understanding of the function of baculovirus genes and how they are regulated during infection is the detailed physical analysis of AcMNPV genes and their products. In this report, we analyzed the size, direction of transcription, and location of the DNA sequences that specify the 5′ and 3′ ends of the mRNA for AcMNPV polyhedrin, the major structural polypeptide of the viral occlusion. S1 nuclease analysis of viral RNA will show that the polyhedrin gene has no detectable intervening sequences. In addition, polyhedrin and its mRNA will be shown to accumulate to high levels in infected cells.

A low-molecular-weight AcMNPV protein with a relative size in sodium dodecyl sulfate polyacrylamide gels of about 10,000 (10K) is produced in infected cells at a level comparable to that of polyhedrin (23). The majority of 10K protein produced in infected cells is nonstructural; however, it is also a very minor structural component of the virion (23). Nothing is known of the function of this viral protein. Here we describe the size and approximate map positions of the transcripts for 10K protein and other major cytoplasmic viral RNAs present at 48 h after infection. With the exception of polyhedrin mRNA, most of these viral RNAs mapped within or near the region of the genome that codes for 10K protein. To facilitate the physical analyses of polyhedrin and 10K genes, we have cloned numerous AcMNPV DNA fragments into bacterial plasmids. A description of these cloned DNA fragments is also presented.

MATERIALS AND METHODS

Cells and virus. *Spodoptera frugiperda* IPLB-SF21 (Sf) cells were maintained at 27°C in Grace's insect tissue culture medium (KC Biologicals, Lenexa, Kans.) containing 10% fetal bovine serum and no antibiotics. The plaque-purified variant of AcMNPV, E2, was used to infect cells at a multiplicity of 20 PFU per cell as described previously (27). AcMNPV extra-

cellular virus, occluded virus, and polyhedrin were purified from infected Sf cells as described previously (8, 22).

Analysis of infected-cell proteins and polyacrylamide gel electrophoresis. Cell proteins were pulse-labeled with 100 μCi of L-[4,5-^{3}H]leucine (60 Ci/mmol; Schwartz/Mann, Orangeburg, N.Y.) per ml and analyzed on 10% polyacrylamide gels as described previously (23). Fluorography was performed as described by Laskey and Mills (10), and the gels were exposed to Kodak X-Omat RP film at −80°C.

Enzymes. All enzymes were obtained from Bethesda Research Laboratories, Rockville, Md., and the reactions were carried out in buffers recommended by the supplier.

Cloning of AcMNPV DNA restriction fragments. *Hin*dIII, *Pst*I, and *Bam*HI DNA restriction fragments of AcMNPV DNA were cloned into the bacterial plasmid pBR322, and AcMNPV *Eco*RI DNA fragments were cloned into the plasmid pBR325. Plasmid DNAs were restricted with either *Hin*dIII, *Pst*I, *Bam*HI, or *Eco*RI, treated with 0.01 U of calf intestinal alkaline phosphatase for 15 min at 37°C, extracted with phenol, precipitated with ethanol, and suspended in distilled water at 1 mg/ml. AcMNPV DNA fragments were mixed with an equimolar quantity of the appropriate vector at a combined concentration of 50 ng/μl in 10 μl of 50 mM Tris-hydrochloride (pH 7.6)–10 mM $MgCl_2$–1.0 mM ATP–15 mM dithiothreitol–1 μg of bovine serum albumin–2 U of T4 DNA ligase, and the reaction was incubated at room temperature for 1 h. Recombinant plasmids were transformed and maintained in *Escherichia coli* RR1 cells (kindly provided by Savio Woo, Baylor College of Medicine, Houston, Tex.).

Competent RR1 cells were prepared by the following procedure (modification of a procedure obtained from Savio Woo). RR1 cells were grown to a density of 0.28 to 0.32 optical density at 550 nm U/ml in 50 ml of Luria broth, centrifuged, washed with 25 ml of ice-cold 5 mM Tris-hydrochloride (pH 7.6)–5 mM $MgCl_2$–0.1 M NaCl, resuspended in 20 ml of ice-cold 5 mM Tris-hydrochloride (pH 7.6)–5 mM $MgCl_2$–250 mM KCl–100 mM $CaCl_2$, and set on ice for 25 min. The cells were sedimented and resuspended in 0.4 ml of the latter buffer. Ten microliters of DNA was added to 100 μl of competent cells and incubated on ice for 60 min. The cells were heat shocked for 2 min at 37°C, 2 ml of Luria broth was added, and the cells were incubated for 30 min at 37°C. A total of 10 to 100 μl of the cell suspension was spread onto agar plates containing 25 μg of ampicillin or tetracycline per ml. From 10^5 to 10^6 antibiotic-resistant colonies per microgram of plasmid DNA were obtained, and about 50% of these had cloned AcMNPV DNA fragments.

Cells containing recombinant plasmids were grown in 1.5 ml of media with 25 μg of ampicillin or tetracycline per ml, and the plasmid DNA was isolated by the rapid boiling method described by Holmes and Quigley (7). Preparative isolation of plasmid DNA was carried out as described previously (7). The identity of the cloned Ac*M*NPV DNA fragments in the recombinant plasmids was determined by restriction enzyme analysis and Southern blot hybridization (21).

All recombinant DNA procedures were carried out according to current National Institutes of Health guidelines.

Restriction map of AcMNPV *Eco*RI-I. To construct a restriction map of AcMNPV DNA fragment *Eco*RI-I, the plasmid containing *Eco*RI-I (pI10) was restricted with one or more restriction enzymes and electrophoresed in agarose gels. To identify which fragments had AcMNPV DNA sequences, DNA fragments were transferred from agarose gels to nitrocellulose (21) and hybridized to AcMNPV [^{32}P]DNA.

Isolation of cytoplasmic RNA. Sf cells in 150-cm^2 cell culture flasks were infected with Ac*M*NPV at a multiplicity of infection of 20 PFU per cell. At 48 h postinfection (p.i.), the cell monolayers were washed twice with cold phosphate-buffered saline and then lysed by adding 2 ml of 30 mM Tris (pH 7.5)–10 mM magnesium acetate–1% Nonidet P-40 directly to each flask. The lysed cells were collected into 50-ml sterile centrifuge tubes and kept on ice with intermittent blending in a Vortex mixer for 15 min. The nuclei were pelleted by centrifugation at 1,500 × *g* for 5 min, and the nuclear RNA was isolated as described previously (8). Cytoplasmic RNA was isolated by adding to the supernatant a 1/10 volume of 2.0 M potassium acetate (pH 5.0) and a 1/10 volume of 4% sodium dodecyl sulfate–0.3 M EDTA and extracting the RNA twice with phenol and twice with chloroform-isoamyl alcohol (24:1). The RNA was precipitated with 2 volumes of ethanol, incubated overnight at −20°C, and then collected by centrifugation.

Polyadenylated [poly(A)$^+$] RNA was isolated by oligodeoxythymidylate-cellulose chromatography as described previously (27).

Transfer of RNA from denaturing gels to nitrocellulose and hybridization. Two micrograms of poly(A)$^+$ RNA or 40 μg of total RNA were denatured at 60°C for 15 min in 50% recrystallized formamide in electrophoresis buffer consisting of 2.2 M formaldehyde–0.02 M morpholinepropanesulfonic acid (Sigma Chemical Co., St. Louis, Mo.) (pH 7.4)–0.005 M sodium acetate–0.001 M EDTA. RNA samples were electrophoresed at 90 V for 12 h in 1.0% agarose gels containing 2.2 M formaldehyde in electrophoresis buffer. RNA was transferred to nitrocellulose filters as described previously (18), except the agarose gels were not treated before transfer. The filters were washed briefly in 4× SSC (1× SSC = 0.15 M NaCl plus 0.015 M sodium citrate), baked at 80°C for 2 h, and incubated in a solution of 50% formamide–5× SSC–200 μg of denatured calf thymus DNA per ml–5.0 μg of poly(A) per ml–2× Denhardts (1× Denhardts = 0.02% [wt/vol] Ficoll–polyvinylpyrolidone–bovine serum albumin) at 43°C for 2 to 4 h and then hybridized with 10^6 cpm of denatured [^{32}P]DNA per ml. After incubating at 41°C for 24 h, the filters were washed sequentially in 2× SSC, 0.1% sodium dodecyl sulfate and 0.1× SSC, and 0.1% sodium dodecyl sulfate at 60°C. Dried filters were exposed to X-ray film with the aid of intensifying screens at −80°C.

S1 nuclease analysis. The procedure of Berk and Sharp (2) as described in detail by Favaloro et al. (6) was carried out by hybridizing poly(A)$^+$ RNA to viral or cloned DNA fragments. After S1 nuclease treatment, the samples were analyzed by electrophoresis on neutral and alkaline agarose gels, and the DNA fragments were transferred to nitrocellulose and hybridized to [^{32}P]DNA (6). A modification of this procedure (28) employing 3′ or 5′ end-labeled AcMNPV DNA restriction fragments was used to map the DNA

sequences that specify the 3′ and 5′ ends of polyhedrin mRNA, respectively. After S1 digestion, end-labeled DNA fragments were electrophoresed in alkaline agarose (2) or 8% polyacrylamide–7 M urea sequencing gels under denaturing conditions (17).

Radiolabeled DNA. Plasmid DNAs were evenly labeled with [^{32}P]dCTP by nick translation (16) to a specific activity of about 5×10^8 cpm/μg of DNA. The 5′ and 3′ ends of DNA restriction fragments were labeled with ^{32}P as described by Weaver and Weissmann (28). To obtain DNA labeled at only one end, end-labeled DNAs were cleaved with an enzyme that cut the fragments asymmetrically, and the appropriate end-labeled fragments were purified from agarose gels as described previously (20).

3′-Specific cDNA probe. A labeled cDNA probe specific for the 3′ ends of cytoplasmic poly(A)$^+$ RNA was made in the following manner. About 1 μg of poly(A)$^+$ RNA isolated from the cytoplasm of AcMNPV-infected cells at 48 h p.i. was partially hydrolyzed at a high pH as described previously (27), and the 3′ ends were purified by binding to an oligodeoxythymidylate-cellulose column. After extensive washing in high salt buffer (27), the truncated poly(A)$^+$ RNA was eluted and precipitated with ethanol. An oligodeoxythymidylate$_{12-15}$ primer (Collaborative Research, Inc., Waltham, Mass.) was hybridized to the truncated RNAs, and [^{32}P]cDNA was synthesized by extension with avian myeloblastosis virus reverse transcriptase in the presence of [α-^{32}P]dCTP and unlabeled nucleotides (14). The length of the cDNA probe was 50 to 250 bases.

RESULTS

Polyhedrin and 10K proteins were detected in infected Sf cells at 15 to 18 and 12 to 15 h p.i., respectively (data not shown). It was shown previously that, at 21 h p.i., 10K protein is the major translation product (23). Here we demonstrated that 10K protein was made at a high level to at least 99 h p.i. (Fig. 1). AcMNPV polyhedrin was also being translated from about 24 to 99 h p.i. at a relatively high level (Fig. 1), and both of these proteins accumulated in cells late in infection (Fig. 2, lane c). Polyhedrin purified from AcMNPV occlusions isolated from infected Sf cells migrated as two distinct bands (Fig. 2, lane d). The reason for this apparent heterogeneity of polyhedrin in these cells is not known.

A minor virion protein of the same mobility in polyacrylamide gels as 10K protein has been observed (23). Also reported is a major low-molecular-weight virus structural protein that has been shown to be very basic (24) and that is 19K in extracellular virus (Fig. 2, lane b) and 18.5K in virions isolated from occlusions (18). The 19K protein was labeled during infection with neither L-[^{3}H]leucine (Fig. 2, lane a) nor L-[^{35}S]methionine or a mixture of L-[^{3}H]-amino acids (23). These differences in the incorporation of labeled amino acids into 10K and 19K proteins is good evidence that 10K protein is not related to the major AcMNPV structural polypeptide. Since 19K protein does not incorporate labeled amino acids during infection, the 19K and 18.5K virion polypeptides may be host proteins. The leucine-deficient 54K virion protein (Fig. 2, lane b) has been discussed previously (23).

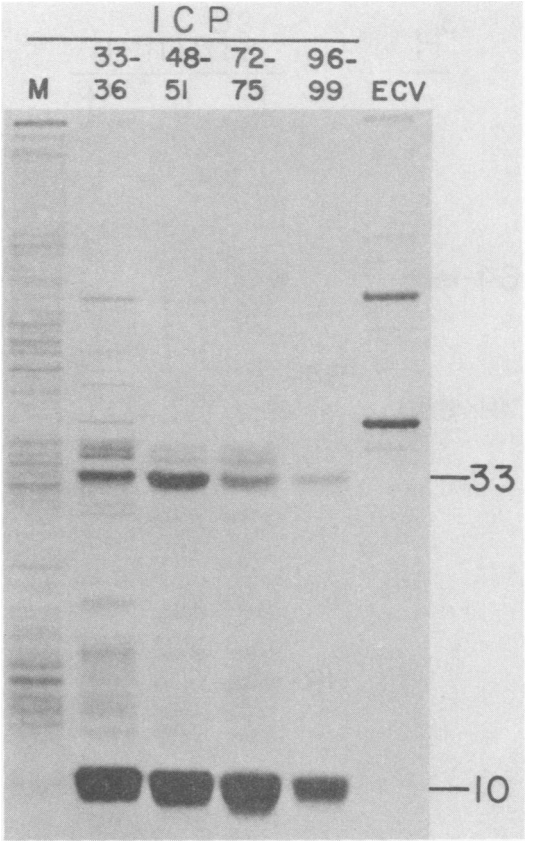

FIG. 1. Pulse-labeled AcMNPV-infected cell proteins. *S. frugiperda* cells were infected with AcMNPV and labeled with L-[^{3}H]leucine at 33 to 36, 48 to 51, 72 to 75, and 96 to 99 h p.i. Mock-infected cell proteins (M) were labeled at 33 to 36 h, and L-[^{3}H]leucine-labeled AcMNPV extracellular virus (ECV) was purified from infected cells labeled at 0 to 48 h p.i. Labeled proteins were electrophoresed in 10% polyacrylamide, prepared for fluorography, and exposed for 4 days at −80°C. The 33K and 10K polyhedrin and 10K protein are indicated by 33 and 10, respectively.

Cloned AcMNPV DNA restriction fragments. AcMNPV *Eco*RI, *Hin*dIII, *Bam*HI, and *Pst*I fragments listed in Table 1 were cloned into *E. coli* plasmids. Each of the recombinant plasmids has one cloned viral DNA fragment, except pPQ1, which contains *Hin*dIII-P and -Q, two fragments that are contiguous on the AcMNPV genome. Cloned fragments were identified by restriction analysis and hybridization of ^{32}P-labeled plasmid DNAs to AcMNPV *Eco*RI, *Hin*dIII, *Bam*HI, and *Xho*I fragments. Each of the cloned DNAs hybridized to the expected

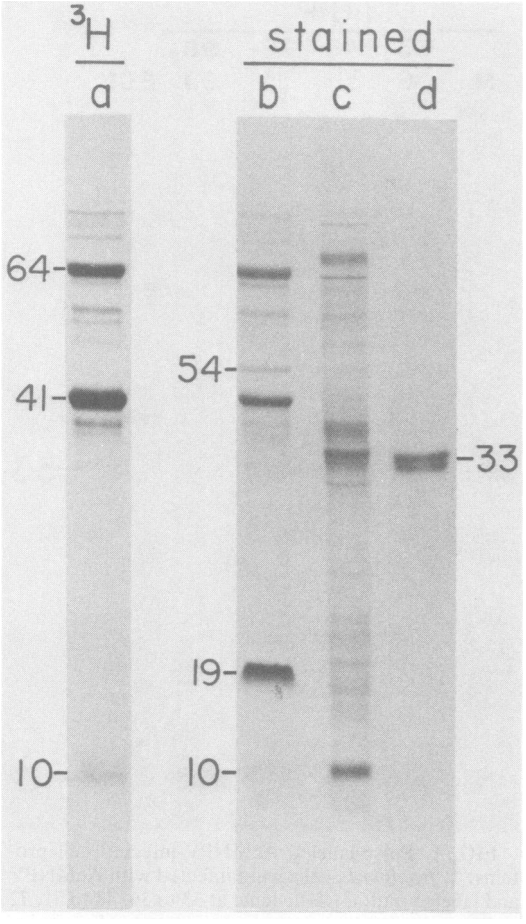

FIG. 2. Comparison of labeled and stained AcMNPV proteins. AcMNPV-infected cells were labeled with L-[^{3}H]leucine from 0 to 48 h p.i., and then the extracellular virus and polyhedrin were purified and a sample of the infected cells was saved. About 30 μg of labeled virus protein (lane b), 50 μg of total infected-cell protein (lane c), and 3 μg of polyhedrin (lane d) were electrophoresed in 10% polyacrylamide and stained with Coommassie blue. A fluorogram exposed for 2 days at −80°C of the virus preparation is also shown (lane a).

AcMNPV viral DNA fragments (data not shown). We also observed homologous sequences present on *Hin*dIII-L and -Q, as reported by Cochran et al. (4). Minor homology was also detected between *Hin*dIII-N, -L, and -A and *Eco*RI-M and between *Eco*RI-C and *Hin*dIII-F. The nature of these intragenomic homologous sequences was not investigated further. However, it is important to consider these sequences when interpreting results from RNA and DNA hybridization experiments.

Restriction map of cloned *Eco*RI-I. Detailed restriction maps were constructed for cloned

TABLE 1. AcMNPV DNA recombinant clones

Fragment	Size (kb[a])	Vector	Number
*Eco*RI			
A	14.2	pBR325	pA154
B	13.3		pB156
C	12.2		pBC2
D	10.4		pD1
E	9.0		pE10
F	8.78		pF20
G	8.70		pG70
H	8.70		pH
I	7.30		pI10
J	6.66		pJ17
K	5.38		pK1
L	3.78		pL
M	3.65		pM1
N	2.38		pN83
O	2.25		pO50
P	1.98		pPQ1
Q	1.93		pPQ5
R	1.14		pR159
S	1.38		pS141
T	1.28		pT7
U or V	0.94		pUV4,5
*Hin*d			
A	21.5	pBR322	pA152
C	11.1		pC
D	9.95		pD
E	9.95		pE
F	8.40		pF151
H	5.60		pH7
I	5.02		pI120
J	4.74		pJ12
K	2.75		pK
L	2.62		pL3
N	2.23		pN63
O	2.23		pO
P and Q	2.10		pPQ1
R	1.79		pR154
S	1.65		pS1
T	1.05		pT10
U	1.02		pU18
V	0.93		pV36
W or X	0.77		pW57
*Pst*I			
B	21.6	pBR322	pB9
D	11.5		pD4
F	8.44		pF14
G	6.91		pG19
H	5.12		pH2
I	4.86		pI20
J	3.45		pJ15
K	3.00		pK17
L	2.90		pL21
M	2.81		pM6
N	2.62		pN24
O	1.66		pO22
*Bam*HI			
D	3.45	pBR322	pDB6
F	1.92		pFB1

[a] kb, Kilobases.

*Eco*RI-I (pI10) (Fig. 3). Plasmid pI10 was digested with one or more enzymes and electrophoresed in 1.0% agarose, and the fragments with AcMNPV DNA sequences were identified by transferring the DNA to nitrocellulose and hybridizing to [^{32}P]AcMNPV DNA (data not shown). All of the enzyme cleavage sites on cloned *Eco*RI-I that had been mapped previously on the AcMNPV genome (4, 19) were in the same relative positions. The sizes of certain fragments and distance between restriction sites were slightly different.

Size and map location of AcMNPV RNAs present at 48 h p.i. Two major viral poly(A)$^+$ RNAs 1,200 and 630 bases in size were detected in cytoplasmic RNA isolated at 48 h p.i., using AcMNPV [^{32}P]DNA as a probe (Fig. 4, lanes A and D). A visual inspection of the RNAs that hybridized to total AcMNPV-labeled DNA (Fig. 4, lanes A and D) suggests that there was an approximately equal amount of the 1,200- and 630-base RNA that together appear to comprise greater than 90% of the total poly(A)$^+$ viral RNA present. Since polyhedrin and 10K protein were the predominant proteins being made in infected cells at 48 h p.i. and the two major RNAs 1,200 and 630 bases were sufficient in size to code for polyhedrin and 10K protein, respectively, we suspected that they specified these proteins. Therefore, similar RNA blots were hybridized to labeled plasmid DNAs that mapped to the region of the DNA where these gene products have been mapped (23, 27). Cloned *Hin*dIII-V (Fig. 4, lane B) and *Eco*RI-I (data not shown) hybridized primarily to the 1,200-base RNA. Cloned *Hin*dIII-F and -T, which are adjacent to *Hin*dIII-V on the genome, did not hybridize to any detectable RNAs. These results are consistent with the fact that RNA selected by *Hin*dII-V is translated in vitro into a 33K protein, but RNA from *Hin*dIII-F and -T is not (23). A longer exposure of the RNA blot probed with *Hin*dIII-V (Fig. 4, lane C) shows that two other minor RNAs also hybridized to *Hin*dIII-V. The relationship of these higher-molecular-weight RNAs to polyhedrin mRNA is not known; however, both were detected in a preparation of nuclear poly(A)$^+$ RNA.

The abundant 630-base RNA hybridized to labeled *Pst*I-B and *Eco*RI-P (Fig. 4, lanes E and

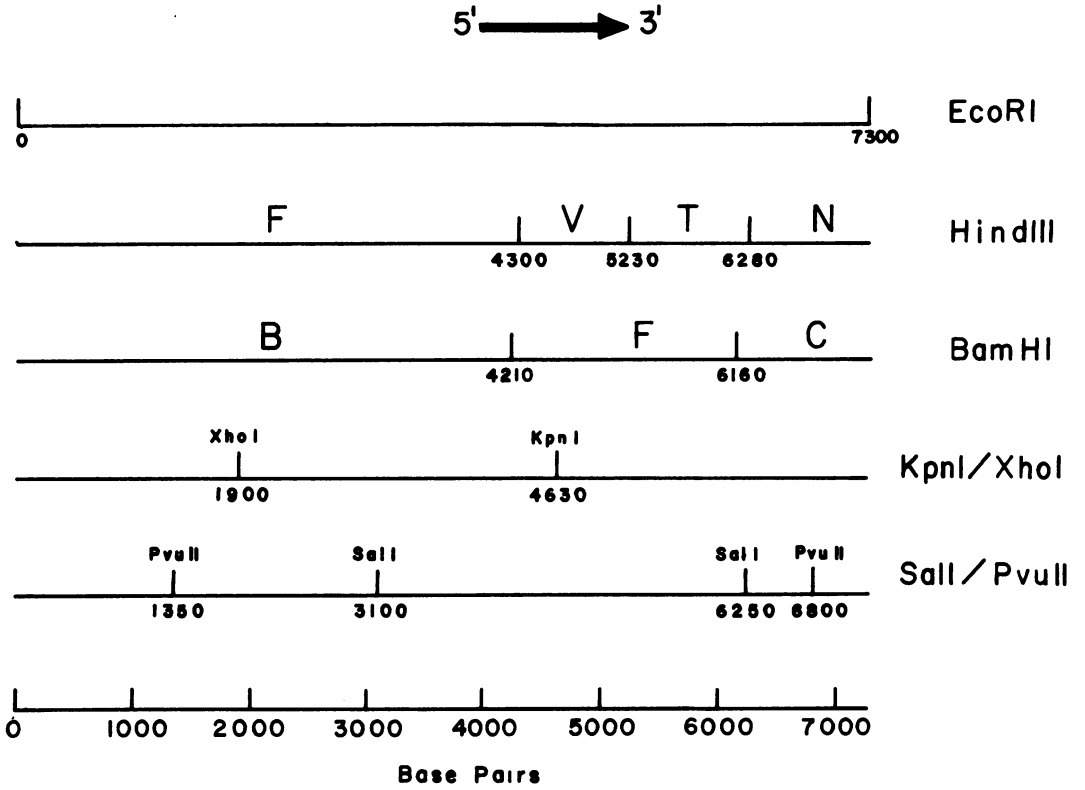

FIG. 3. Restriction map of cloned AcMNPV *Eco*RI-I and the location of the polyhedrin gene. The location of restriction sites are labeled in base pairs. The arrow shows the approximate location of the polyhedrin gene and the direction of transcription.

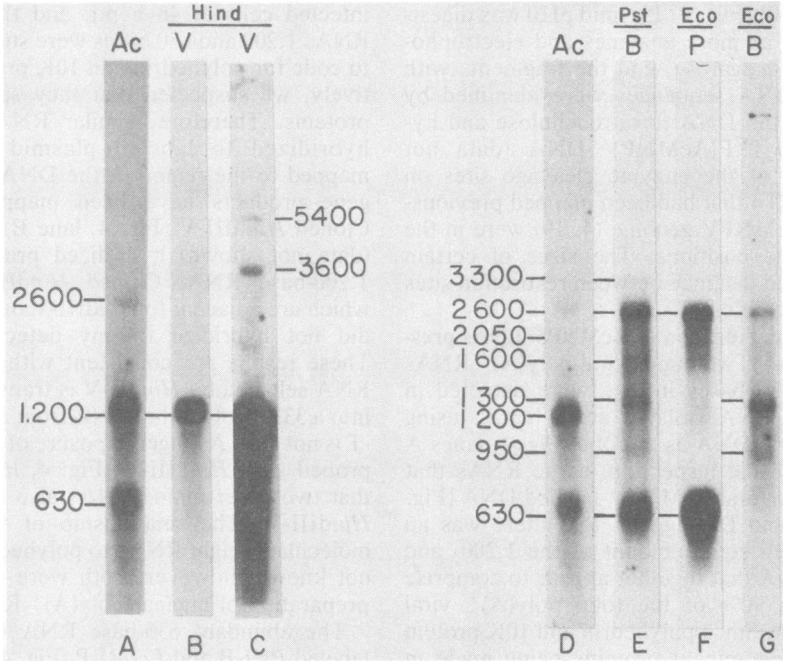

FIG. 4. RNA blot analysis of AcMNPV RNA. Poly(A)$^+$ RNA was isolated from the cytoplasm of AcMNPV-infected cells at 48 h p.i. and denatured with formaldehyde. Samples of 2 μg were electrophoresed in a 1% agarose–2.2 M formaldehyde gel, transferred to nitrocellulose, and hybridized to ^{32}P-labeled AcMNPV (lanes A and D) or cloned DNA fragments *Hin*dIII-V (lanes B and C), *Pst*I-B (lane E), *Eco*RI-P (lane F), and *Eco*RI-B (lane G). Lanes A, B, D, E, F, and G were exposed for 6 h, and lane C was exposed for 48 h at −80°C with the aid of an intensifying screen. The sizes of AcMNPV RNAs are given in nucleotides and were determined relative to *E. coli* 5S, 16S, and 23S ribosomal RNA standards.

F). *Hin*dIII-P hybridized with a 630-base RNA, but the adjacent fragments Q and G did not (data not shown). Since this mapped the 630-base RNA to a region of the AcMNPV genome where 10K maps (23) and 10K was a major protein being made in infected cells at 48 h p.i., this RNA was probably the transcript for 10K. Viral RNAs of 3,300, 2,600, and 2,050 bases were consistently detected in cytoplasmic poly(A)$^+$ RNA isolated at 48 h p.i. when total AcMNPV DNA was used as a probe (Fig. 4, lanes A and D). The 3,300- and 2,600-base RNAs, as well as several additional RNAs not detected on RNA blots, also mapped to AcMNPV *Pst*I-B (Fig. 4 and 5) when total AcMNPV DNA was used as a probe. The 2,050-base viral RNA was the only RNA detected with AcMNPV DNA probe that did not map within either *Eco*RI-I or *Pst*I-B. A summary of the RNA hybridization data is shown in Fig. 5. AcMNPV transcripts of 1,600, 2,600, and 3,300 bases may include the homologous viral sequences present in *Hin*dIII-Q and -L; therefore, one or more of these may have originated from the region of the genome around *Hin*dIII-L.

Hybridization of ^{32}P-labeled cDNA. Cytoplasmic poly(A)$^+$ RNA was isolated at 48 h p.i. and partially hydrolyzed. The truncated 3′ ends were purified and then used as a template for cDNA synthesis. The cDNA probe, specific for the 3′ ends of the RNA, hybridized primarily to two regions of the genome that include the AcMNPV fragments *Hin*dIII-P and -V (Fig. 6, lanes 1 through 4). These results are consistent with the RNA blotting data that mapped the two abundant viral RNAs present in the cytoplasm of infected cells at 48 h p.i. to these two regions.

To locate the DNA fragments homologous to the 3′ end of polyhedrin gene, the ^{32}P-labeled cDNA was hybridized to cloned *Eco*RI-I, *Bam*HI-F, and *Hin*dIII-V plasmid DNA that had been restricted with two or more enzymes and then electrophoresed and transferred to nitrocellulose filters. As expected, the probe hybridized to *Bam*HI-F (Fig. 6, lane 5) and to *Hin*dIII-V (Fig. 6, lane 6). *Kpn*I cleaves both of these fragments once to give two subfragments, respectively labeled F_1, F_2 and V_1, V_2 in Fig. 6. The cDNA probe hybridized exclusively to F_1 and V_1, both of which map to the right of the *Kpn*I site at position 4630 (Fig. 3). Therefore, the 3′ end of the 1,200-base polyhedrin RNA

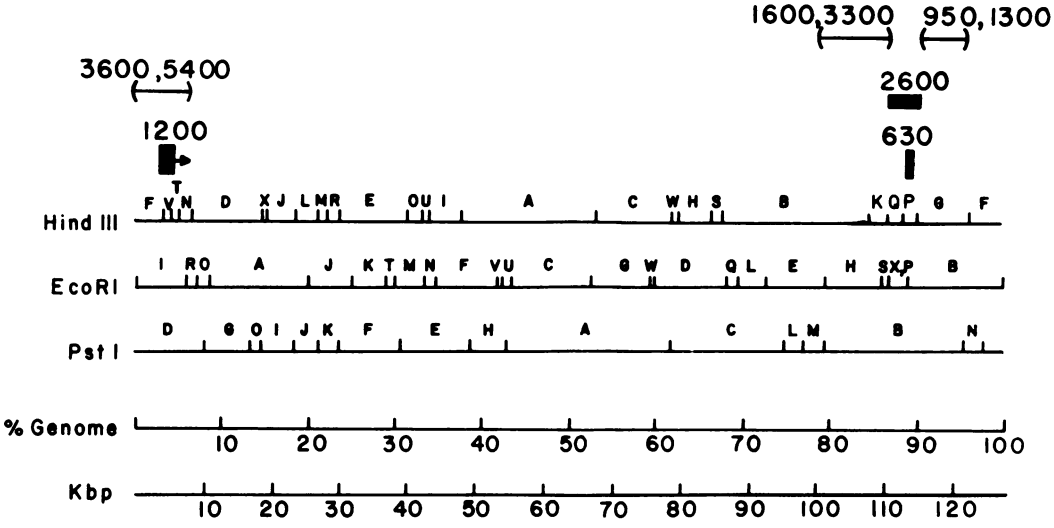

FIG. 5. Approximate location of DNA sequences complementary to the major AcMNPV poly(A)$^+$ cytoplasmic RNAs present in infected cells at 48 h p.i. The width of the lines reflects the relative abundance of the particular RNA in the infected cells, and the sizes are indicated in nucleotides. The 1,200-base RNA was identified as the polyhedrin mRNA, and the direction of transcription is indicated by the arrow. The 630-base RNA was identified as the mRNA for AcMNPV 10K protein. The AcMNPV DNA restriction map is as described previously (2, 16).

maps between positions 4600 and 5200 in *Eco*RI-I (Fig. 3). Since *Hin*dIII-T did not hybridize to polyhedrin mRNA, the 5′ end of the gene is most likely in the other direction in *Hin*dIII-F. S1 nuclease mapping confirmed this conclusion.

The location of the 3′ end of the 630-base RNA was more difficult to determine, as there were six minor RNAs that mapped near the gene sequences for this RNA. Because the 630-base RNA was much more abundant than the minor RNAs, the hybridization of labeled cDNA should still have been relatively specific and was worth considering. *Xho*I-K, *Bam*HI-B, *Eco*RI-B, and *Hin*dIII-P hybridized to the cDNA probe (Fig. 6, lanes 1, 2, 3, and 4, respectively); therefore, the 3′ end of the 630-base RNA was probably located within *Eco*RI-P to the right of the *Hin*dIII site between *Hin*dIII-Q and -P (Fig. 5). This was consistent with the homology detected between *Hin*dIII-P and the 630-base RNA as well as previous mapping data of 10K protein (23).

S1 nuclease analysis of polyhedrin mRNA. Poly(A)$^+$ cytoplasmic RNA was hybridized to a molar excess of AcMNPV or cloned fragments *Eco*RI-I, *Hin*dIII-F, -V, and -T, and *Bam*HI-F, treated with S1 nuclease, and electrophoresed in neutral and alkaline agarose gels (2, 6). A DNA fragment of about 1,200 bases was protected from S1 digestion and detected in alkaline agarose gels when viral RNA was hybridized to *Eco*RI-I (Fig. 7) or total AcMNPV DNA (data not shown). A single 1,200-base DNA fragment was also observed when similar samples were electrophoresed in neutral gels. Most, if not all, of the 930 bases of the coding strand in *Hin*dIII-V were protected from S1 digestion by polyhedrin RNA (Fig. 7), and about 1,000 bases of *Bam*HI-F were protected (data not shown). Therefore, the polyhedrin mRNA hybridized to a contiguous sequence of DNA of about 1,200 bases extending from approximately 4,000 to 5,200 bases from the origin of the AcMNPV DNA map (Fig. 3).

The specificity of the cDNA probe specific for the 3′ end of polyhedrin mRNA indicated that transcription would be from left to right and the 5′ end of the gene would be near position 4000 in *Eco*RI-I. To confirm this indication and to map more precisely the location of the gene sequences that specify the 5′ and 3′ ends of polyhedrin mRNA, we used the Weaver and Weissmann (28) modification of the S1 nuclease mapping procedure. *Eco*RI-*Hin*dIII fragment F and *Eco*RI-*Bam*HI fragment B in cloned *Eco*RI-I were 5′ end labeled in such a way that only one of the ends at positions 4300 and 4210 (Fig. 3) were labeled. The labeled fragments were purified from agarose gels, hybridized to poly(A)$^+$ RNA, digested with S1 nuclease, and then electrophoresed in a 7 M urea–8% polyacrylamide sequencing gel under denaturing conditions (17). Two major bands of 303 and 310 ± 2 and 213 and 220 ± 2 bases, respectively, and several minor bands were protected from S1 nuclease (Fig. 8). These results suggest that polyhedrin mRNA

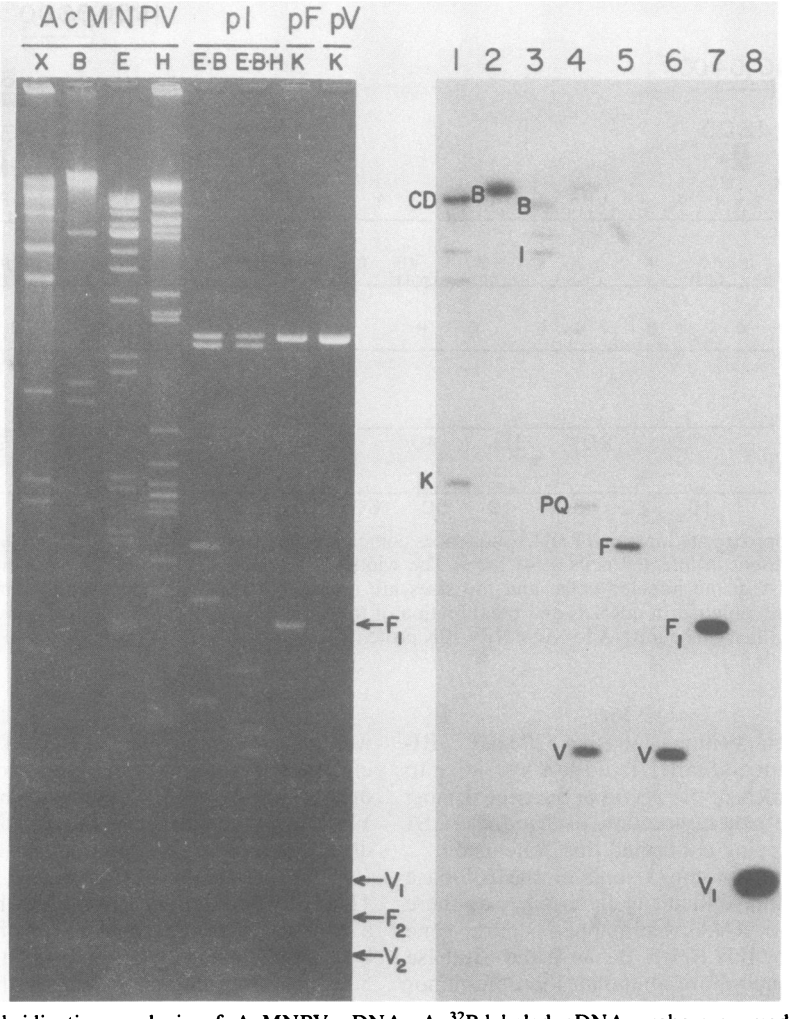

FIG. 6. Hybridization analysis of AcMNPV cDNA. A ^{32}P-labeled cDNA probe was made by primed synthesis with avian myeloblastosis virus reverse transcriptase, using the 3′ ends of AcMNPV poly(A)$^+$ RNA isolated from infected cells at 48 h p.i. as a template. The cDNA probe was hybridized to AcMNPV DNA (right panel) restricted with *Xho*I (X), *Bam*HI (B), *Eco*RI (E), and *Hin*dIII (H) and to cloned plasmids *Eco*RI-I (pI) restricted with *Eco*RI and *Bam*HI (E · B) or *Eco*RI, *Bam*HI, and *Hin*dIII (E · B · H); cloned *Bam*HI-F (pF) restricted with *Bam*HI and *Kpn*I (K); and cloned *Hin*dIII-V (pV) restricted with *Hin*dIII and *Kpn*I (K) (lanes 1 through 8, respectively). *Kpn*I subfragments of AcMNPV *Bam*HI-F (F_1 and F_2) and *Hin*dIII-V (V_1 and V_2) are marked. An ethidium bromide-stained gel of these fragments is shown in the left panel. Certain fragments discussed in the text are labeled.

may be a mixture of two major RNA species whose 5′ ends map approximately at positions 3997 and 3990 (Fig. 3). It is also possible that the first 5 to 10 bases of the polyhedrin RNA-DNA hybrid were sensitive to S1 digestion. We are using primer-extension and RNA sequencing methods to help answer this question.

The 3′ end of the gene was mapped in a similar manner, using a 3′ end-labeled *Kpn*I-*Eco*RI fragment extending from 4,630 to 7,300 base pairs in *Eco*RI-I and 3′ end-labeled *Hin*dIII-V. Results obtained from alkaline agarose gels (data not shown) mapped DNA sequences that specify for the 3′ end of polyhedrin mRNA near the *Hin*dIII site at 5,230 ± 50 bases (Fig. 3).

DISCUSSION

In this study, we demonstrated that two AcMNPV genes, polyhedrin and 10K protein, were expressed at high levels at about 48 h p.i. and were continually expressed to at least 99 h p.i. At 48 h p.i., the mRNA and the protein from these two genes had accumulated in infected

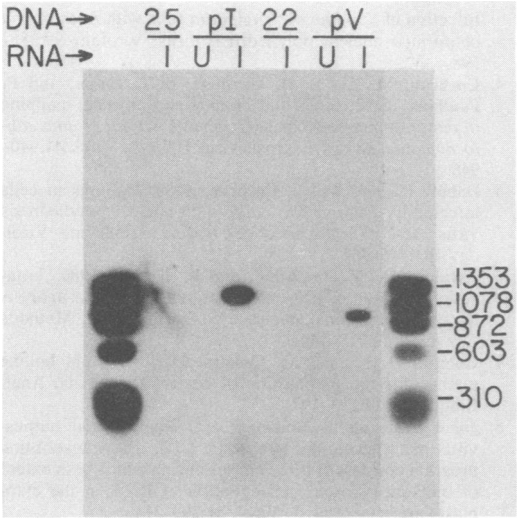

FIG. 7. S1 nuclease analysis of polyhedrin mRNA. Poly(A)$^+$ RNA isolated from AcMNPV-infected (I) cells at 48 h p.i. or uninfected (U) cells were hybridized to cloned *Eco*RI-I (pI) and *Hin*dIII-V (pV) DNA. As controls, pBR325 (25) and pBR322 (22) DNAs were also hybridized to infected-cell RNA. The preparations were hybridized for 3 h in 80% formamide (2) at 50°C, diluted, and digested with 500 U of S1 nuclease per ml at 37°C for 15 min. The RNA-DNA hybrids were precipitated with alcohol, denatured, and electrophoresed in 1.2% alkaline agarose. The DNA was transferred to nitrocellulose and hybridized with ^{32}P-labeled *Eco*RI-I. φX174 *Hae*III restriction fragments were electrophoresed in the first and last lanes, transferred, and detected by adding ^{32}P-labeled φX174 DNA to the hybridization mix. The size of φX174 DNA fragments in nucleotides is indicated.

cells. In a recent study (J. M. Vlak and S. van der Krol, Virology, in press), it was demonstrated that, at 24 h p.i. in AcMNPV-infected cells, there is an abundance of cytoplasmic poly(A)$^+$ RNA that hybridizes to the regions of the genome where polyhedrin and 10K genes are located. The DNA sequences responsible for the preferred expression of polyhedrin and 10K protein late in infection are of considerable interest and can be used as a model to study the control of gene expression in insect cells. In addition, the copious quantities of RNA and protein made from these genes will facilitate such genetic studies. The necessity for the production of large amounts of polyhedrin to serve as the major component of viral occlusions in AcMNPV-infected cells is obvious. The reason for the production of a large quantity of 10K protein and its continued synthesis several days after infection is not readily apparent. A small amount of 10K protein is most likely required during the first 24 to 36 h p.i., when virions are being assembled, because the evidence is that 10K protein is a minor component of the virus particles, but the majority of 10K protein made in infected cells is apparently nonstructural. Since 10K protein is a late gene product and is produced at high levels very late in infection, it may also be involved in the occlusion process.

The mRNA for AcMNPV polyhedrin was measured in formamide gels as being about 1,200 bases in size. This is most likely an underestimate, as the gene for AcMNPV polyhedrin was

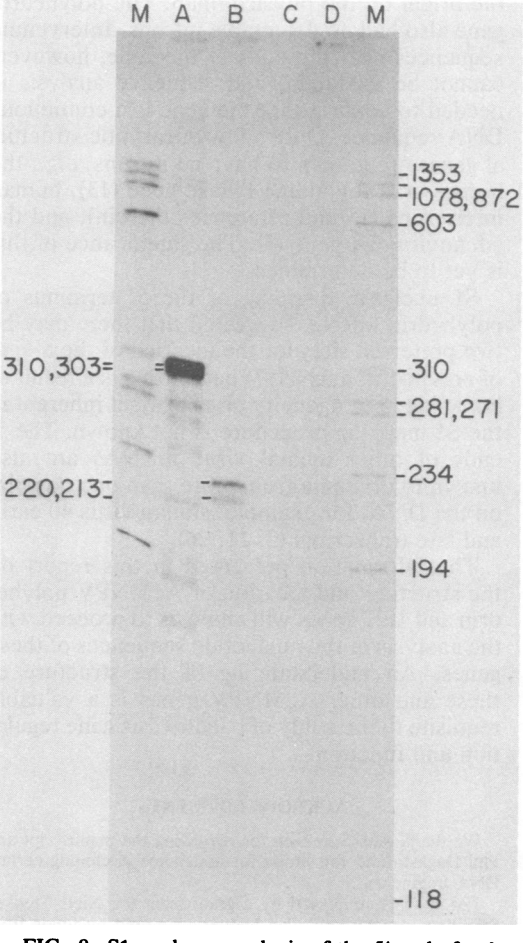

FIG. 8. S1 nuclease analysis of the 5′ end of polyhedrin mRNA. To locate the 5′ end of polyhedrin mRNA, AcMNPV DNA fragments were 5′ end labeled at positions 4300 and 4210 (Fig. 3) and hybridized with infected-cell RNA (lanes A and B, respectively) or uninfected cell RNA (lanes C and D, respectively). The preparations were treated with S1 nuclease as described in the legend to Fig. 7, precipitated with alcohol, denatured by boiling for 3 min in 99% formamide, and electrophoresed in a 0.4-mm 8% polyacrylamide–7 M urea gel at 2,000 V for 2 h. φX174 *Hae*III fragments labeled at the 5′ ends were included as size markers (M). The gel was exposed to X-ray film at −80°C for 24 h.

determined from S1 nuclease experiments to be about 1,200 bases and the polyhedrin mRNA is also poly(A)$^+$ (27). In the first report to describe the isolation of AcMNPV polyhedrin mRNA (25), it is speculated that an abundant RNA of about 650 bases is the polyhedrin mRNA. It is likely that this RNA is the 10K mRNA and, as suggested by Vlak and Krol (in press), the polyhedrin mRNA is larger. The 5′ end of the AcMNPV polyhedrin gene was located at approximately 3,990 base pairs, and the 3′ end was located at about 5,200 base pairs to the right of the origin on the physical map. The polyhedrin gene also had no detectable introns. Intervening sequences near the ends of the gene, however, cannot be excluded, and sequence analysis is needed to confirm that the gene is a continuous DNA sequence. Only a few eucaryotic structural genes are known to have no introns, e.g., the herpesvirus thymidine kinase gene (13), human interferons (15 and references therein), and the adenovirus IX gene (1). The significance of this is yet to be determined.

S1 nuclease mapping of the 5′ terminus of polyhedrin mRNA suggested that there may be two preferred sites for the location of the 5′ end of polyhedrin mRNA. Whether this is a result of bona fide heterogeneity or an artifact inherent in the S1 mapping procedure is not known. The 5′ ends of other animal virus mRNAs are also known to originate from more than one position on the DNA, for example, simian virus 40 early and late transcripts (2, 11, 26).

The information presented in this report on the structure and location of AcMNPV polyhedrin and 10K genes will allow us to proceed with the analysis of the nucleotide sequences of these genes. An understanding of the structure of these and other AcMNPV genes is a valuable requisite to the study of baculovirus gene regulation and function.

ACKNOWLEDGMENTS

We thank Ann Sorensen for reviewing the manuscript and Phil Dagasto and Jan Stoltz for assistance in cloning certain DNA fragments.

J.M.V. was supported by a grant from the Niels Stensen Stichting, Amsterdam, The Netherlands, and by funds from Texas A&M University and the Texas Agricultural Experiment Station. This work was supported in part by Public Health Service grant AI 14755-13A1 from the National Institutes of Health and by Texas Agricultural Experiment Station Project TEXO 6316.

LITERATURE CITED

1. **Alestrom, P., G. Akusjarvi, M. Perricaudet, M. B. Mathews, D. F. Klessig, and U. Pettersson.** 1980. The gene for polypeptide IX of adenovirus type 2 and its unspliced messenger RNA. Cell **19**:671–681.
2. **Berk, A. J., and P. A. Sharp.** 1978. Spliced early mRNA of simian virus 40. Proc. Natl. Acad. Sci. U.S.A. **75**:1274–1278.
3. **Carstens, E. B., S. T. Tijia, and W. Doerfler.** 1979. Infection of *Spodoptera frugiperda* cells with *Autographa californica* nuclear polyhedrosis virus. Virology **99**:386–398.
4. **Cochran, M. A., E. B. Carstens, B. T. Eaton, and P. Faulkner.** 1982. Molecular cloning and physical mapping of restriction endonuclease fragments of *Autographa californica* nuclear polyhedrosis virus DNA. J. Virol. **41**:940–946.
5. **Dobos, P., and M. A. Cochran.** 1980. Proteins in cells infected by *Autographa californica* nuclear polyhedrosis virus (Ac-NPV): the effect of cytosine arabinoside. Virology **103**:446–464.
6. **Favaloro, J., R. Treisman, and R. Kamen.** 1980. Transcription maps of polyoma virus-specific RNA: analysis by two dimensional nuclease S1 gel mapping. Methods Enzymol. **65**:718–749.
7. **Holmes, D. S., and M. Quigley.** 1981. A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. **114**:193–197.
8. **Jones, P. C., and B. Roizman.** 1979. Regulation of herpesvirus macromolecular synthesis. VIII. The transcription program consists of three phases during which both extent of transcription and accumulation of RNA in the cytoplasm are regulated. J. Virol. **31**:299–314.
9. **Kelly, D. C., and T. Lescott.** 1980. Baculovirus replication: protein synthesis in *Spodoptera frugiperda* cells infected with *Trichoplusia ni* nuclear polyhedrosis virus. Microbiologica **4**:35–57.
10. **Laskey, R. A., and A. D. Mills.** 1975. Quantitative film detection of ^{3}H and ^{14}C in polyacrylamide gels by fluorography. Eur. J. Biochem. **56**:335–341.
11. **Lai, C.-J., R. Dhar, and G. Khoury.** 1978. Mapping the spliced and unspliced late lytic SV40 RNAs. Cell **14**:971–982.
12. **Maruniak, J. E., and M. D. Summers.** 1981. *Autographa californica* nuclear polyhedrosis virus phosphoproteins and synthesis of intracellular proteins after virus infection. Virology **109**:25–34.
13. **McKnight, S. L.** 1980. The nucleotide sequence and transcript map of the herpes virus thymidine kinase gene. Nucleic Acids Res. **24**:5949–5963.
14. **McReynolds, L. A., J. F. Catterall, and B. W. O'Malley.** 1977. The ovalbumin gene: cloning of a complete ds-cDNA in a bacterial plasmid. Gene **2**:21–231.
15. **Ohno, S., and T. Taniguchi.** 1981. Structure of a chromosomal gene for human interferon B. Proc. Natl. Acad. Sci. U.S.A. **78**:5305–5309.
16. **Rigby, P. W. J., M. Diechmann, C. Rhodes, and P. Berg.** 1977. Labeling DNA to high specific activity in vitro by nick translation with DNA polymerase I. J. Mol. Biol. **133**:237–251.
17. **Sanger, F., and A. R. Coulson.** 1978. The use of thin acrylamide gels for DNA sequencing. FEBS Lett. **87**:107–110.
18. **Smith, G. E., and M. D. Summers.** 1978. Analysis of baculovirus genomes with restriction endonucleases. Virology **89**:517–527.
19. **Smith, G. E., and M. D. Summers.** 1979. Restriction maps of five *Autographa californica M*NPV variants, *Trichoplusia ni M*NPV, and *Galleria mellonella M*NPV DNAs with endonucleases *Sma*I, *Kpn*I, *Bam*HI, *Sac*I, *Xho*I, and *Eco*RI. J. Virol. **30**:828–838.
20. **Smith, G. E., and M. D. Summers.** 1980. Restriction map of *Rachiplusia ou* and *Rachiplusia ou-Autographa californica* baculovirus recombinants. J. Virol. **33**:311–319.
21. **Smith, G. E., and M. D. Summers.** 1980. The bidirectional transfer of DNA and RNA to nitrocellulose or diazobenzyloxymethyl paper. Anal. Biochem. **109**:123–129.
22. **Smith, G. E., and M. D. Summers.** 1981. Application of a novel radioimmunoassay to identify baculovirus structural proteins that share interspecies antigenic determinants. J. Virol. **39**:125–137.
23. **Smith, G. E., J. M. Vlak, and M. D. Summers.** 1982. In vitro translation of *Autographa californica* nuclear polyhedrosis virus early and late mRNAs. J. Virol. **44**:199–

208.
24. **Tweeten, K. A., L. A. Bulla, Jr., and R. A. Consigili.** 1980. Characterization of an extremely basic protein derived from granulosis virus nucleocapsids. J. Virol. **33**:866–876.
25. **van der Beek, C. P., J. D. Saaijer-Riep, and J. M. Vlak.** 1980. On the origin of the polyhedral protein of *Autographa californica* nuclear polyhedrosis virus. Virology **100**:326–333.
26. **Villarreal, L. P., R. T. White, and P. Berg.** 1979. Mutational alterations within the simian virus 40 leader segment generate altered 16S and 19S mRNAs. J. Virol. **29**:209–219.
27. **Vlak, J. M., G. E. Smith, and M. D. Summers.** 1981. Hybridization selection and in vitro translation of *Autographa californica* nuclear polyhedrosis virus mRNA. J. Virol. **40**:762–771.
28. **Weaver, R. F., and C. Weissmann.** 1979. Mapping of RNA by a modification of the Berk-Sharp procedure: the 5′ termini of 15 S B-globin mRNA precursor and mature 10 S B-globin mRNA have identical map coordinates. Nucleic Acids Res. **7**:1175–1193.
29. **Wood, H. A.** 1980. *Autographa californica* nuclear polyhedrosis virus-induced protein in tissue culture. Virology **102**:21–27.